MAKERERE UNIVERSITY

SEROTYPING AND ANTIMICROBIAL RESISTANCE PATTERNS OF *SALMONELLA* FROM PORK OUTLETS IN KAMPALA

A DISSERTATION SUBMITTED TO MAKERERE UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE IN INFECTIOUS DISEASE MANAGEMENT OF MAKERERE UNIVERSITY KAMPALA

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DECLARATION

I, Dickson **NDOBOLI,** declare that the work presented in this report is original and has never been submitted to any University or institution for academic gain.

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DEDICATION

I dedicate this work to my mentors Kristina Roesel, Stephan Huehn and Martin Heilmann who has continually urged me to upgrade my academic qualification, experience and career.

To my dear grandmother Agaali Nalwaya who constantly believes in me and prays for my success, and last but not least, my wonderful wife Nangendo Joanita for the intellectual support rendered during this working period.

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ACRONYMS AND ABBREVIATIONS

BA	Blood agar
CDC	Center for Disease Prevention and Control
CDL	Central Diagnostic Laboratory
COVAB	College of Veterinary medicine Animal resources and Biosecurity
FAO	Food and Agriculture Organisation
FUB	Freie University of Berlin
MHA	Mueller Hinton agar
Ml	milliliter
NA	Nutrient agar
NCCLS	National Committee for Clinical Laboratory Standards
NTS	Non-Typhoidal Salmonella
PFGE	Pulsed-Field Gel Electrophoresis
spp.	Species
ssp.	Sub-species
ISO	International Standards Organisation

ABSTRACT

Introduction: This study was done as part of the safe food fair project of the international livestock research institute. The study followed two earlier studies; the first one by Kung et al who mapped all pork joints (small to medium size restaurants selling mainly ready to eat pork and raw pork prepared at home) in Kampala district and his study was followed by Martin Heilmanns study who studied food hygiene in pork restaurants in kampala using salmonella as an indicator pathogen. This study is a follow up study of the isolated salmonella to determinethe serotypes of salmonella isolates, the antimicrobial susceptibility pattern and presence of shared plasmid**.** A total 674 samples were collected from 77 pork restaurants mapped by Kungu et al, of these 55 (8%) were confirmed as *Salmonella Enterica subspecies Enterica* according to ISO 6579 by Martin et al. The 55 isolates were in this study serotyped using biochemical characterization and later using O and H antigen antisera's. Antimicrobial susceptibility testing was done by disc diffusion method according to Kirby buer utilizing 22 antibiotic antibiotic discs and plasmid profile analysis was done using Incompatibility typing method according to Carrotelli et al. Seven different serovars were identified: *S Enterica. Enteritidis S.Enterica Offa. Arechavaleta, S. Gallinarum , S. Zanzibar , S. Kampala,* and *S. Saintpaul* . Multidrug resistance (resistance to more than one antibiotic) was found in 54/55 (98%) in the strains collected. Six incompatibility plasmid profile groups (FIA, W, FIC, FIB, P, and Y) were identified in 54/55 98.2% of the samples. Seven Salmonella serovars **(**Enteritidis, Gallinarum, Arechavaleta, Zanzibar, Kampala, and Saintpaul**)** were identified in this study, majority of them were resistant to more than one antibiotics. A total of 6 shared plasmids were identified among the 55 *Salmonella Enterica*.

CHAPTER ONE

1.0. INTRODUCTION

1.1 Background

The term pork outlets (Joints, butchers, restaurants) refers to small- to medium- size restaurants serving ready-to-eat pork for consuming on site as well as raw pork that is prepared at home (Heilmann et al., 2015). Pork joints are found along busy trading centers and streets in the suburbs of Kampala. In most cases pork joints are semi-permanent or permanent wooden stalls that are open from morning till late night, where clients' gather to enjoy roasted or fried pork, often served with alcoholic drinks (Han et al., 2013; Grace et al., 2014). Prepared pork meals are always served along with fresh accompaniments such as onions, tomatoes, cabbage, green peppers and avocado, usually chopped in very small pieces to form what is locally known as 'kachumbari'. Food preparation is done under poor hygienic conditions. Flies are common, and pork is left out on a table or hanging in an open window to attract clients (Heilmann et al., 2015). These factors in combination with daily temperatures ranging between 20°C and 37°C, especially during the hot months from March to September, expose pork to bacterial contamination (Heilmann et al., 2015).

In general, microbiological contamination of food is a major public health concern. The first global assessment of foodborne disease found that 79% of the burden was due to microbes (Havelaar et al., 2015). Food can be contaminated during production or along the value chain mostly due to poor hygienic practices (Mensah et al., 2002). Common causes of microbial food-borne infection include: non typhoidal Salmonella (NTS), *S. Typhi, Vibrio cholerae, toxigenic Escherichia (E.) coli, Campylobacter species (spp.), norovirus,* and *Listeria monocytogenes* (Annan-Prah et al.,

2011; Havelaar et al., 2015; Mensah et al., 2002; Tambekar et al., 2011; Wawa et al., 2009). Among the 31 foodborne hazards investigated by the WHO Foodborne Disease Burden Epidemiology Reference Group (FERG), NTS was the most important in terms of overall burden and deaths (Havelaar et al., 2015).

Cases of drug-resistant *Salmonella spp.* have been reported in a number of countries in East Africa (Kariuki et al., 2006; Kikuvi et al., 2010; Omulo et al., 2015) and are likely to increase (Byarugaba., 2004; Sirinavin et al., 2004). Previous studies in Uganda show a high prevalence of multi-resistant strains, evidence of cross-species transmission of plasmids, and drug resistance, between animals and humans (Kalule et al., 2012; Tinega et al., 2016). *Salmonella spp.* are important causes of food-borne diseases, and typing methods such as plasmid profiling, ribotyping and Pulsed-Field Gel Electrophoresis (PFGE) have been used to aid diagnosis and outbreak investigation (Lukinmaa et al., 2004).

In a community-based prevalence survey at pork outlets in Kampala, *Salmonella enterica sub-species (ssp.) enterica* was investigated as an indicator pathogen, potentially carried onto food products by flies (Heilmann et al., 2016). While the prevalence data is presented elsewhere (Heilmann et al., 2015), the aim of this study was to characterize serotypes, phenotypic patterns of antimicrobial resistance and plasmid profiles of the obtained Salmonella isolates.

1.1 Problem statement

Antimicrobial Resistance (AMR) is a global pandemic that has posed threat to public health and food industry especially in animal source foods; majorly due to foodborne illness resulting from *Salmonella*, *Campylobacter*, *Escherichia Coli* among others. Data has been published on *Salmonella* in Uganda however most of it has concentrated on culture and sensitivity often with a

limited number of antibiotics involved in most studies. However food safety objectives from regulatory agencies encourage food industry manufacturers to establish full identification of *Salmonella* serotype to assist with traceability in the food processing (Havelaar et al., 2015). Consumption of pork in Kampala is growing social event often happening in the local restaurants referred to as pork joints, presence of salmonella in these areas is a potential public health risk for foodborne illnesses. The capability to serotype or fingerprint is of importance for surveillance, inspection, and investigation of outbreaks therefore this study will provide information about circulating *Salmonella* serovars in kampala, the pattern of antimicrobial resistance using a broad range of antibiotics and the plasmid Profile. This information will guide public health interventions and aid further research in the management of *Salmonellosis.*

1.2 Justification

Pork joints are growing social hung out place for many city dwellers in Kampala, presence of *Salmonella* in such a public eating place is a potential source of food borne illness. Therefore this study has shown the possibility of foodborne infection from eating at pork outlet in Kampala. The capability to serotype or fingerprint is of importance for surveillance, inspection, and investigation of outbreaks therefore this study has provided information on the circulating *Salmonella* serovars in Kampala, and has given a pattern of antimicrobial resistance using a broad range of antibiotics and information on the presence of shared plasmid among these salmonella isolates. This information has been published to guide public health intervention and aid further research on the management of Salmonellosis.

1.3 Study Objectives

1.3.1 Main objective

To determine the serotypes, antimicrobial resistance profiles and plasmid profiles of 55 *Salmonella* isolates obtained from a study on pork restaurants/joints in Kampala.

1.3.2 Specific objectives

1. To determine the serotypes of *Salmonella* isolates obtained from pork outlets in peri-urban Kampala
2. To define the antimicrobial susceptibility profiles of the different *Salmonella* isolates obtained from pork outlets in Peri Urban Kampala
3. To establish the presence of shared plasmids in the 55 different isolates

1.4 Research question

Is the plasmid profile the same or different in the different isolates?

Is the resistance profile the same or different in these isolates?

Are all isolates of the same or different serovars?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Properties of Salmonella organism

Salmonella are facultative anaerobic, gram negative, small motile rods, (Kyriakides et al., 2008; Meneses et al., 2010; Petry D., 2013). Temperature for growth ranges from 8°C to 45°C, strains can stand between pH (4-9), and is able to grow at water activities above 0.94. Salmonella is heat labile hence the organism can be inactivated at ordinary cooking temperatures (>70°C) although the cooling time and values for temperature and time could change depending on the serotype and the food matrix. In addition, *Salmonella* has been shown to tolerate up to 20% salt concentration (Guthrie et al., 1991). Under freezing conditions (from -23°C to -18°C) this microorganism is able to survive as long as seven years (Kyriakides et al., 2008). The difficulty in controlling *Salmonella* is due to its ability to survive extreme environmental conditions (Guthrie et al., 1991).

Biochemically *Salmonella* are able to reduce nitrates to nitrites, produce gas from glucose (not always), produce hydrogen sulfide on triple-sugar iron agar, and they are usually able to use citrate as the sole carbon source (Molbak et al., 2006). *Salmonella* can be further subdivided by phage typing, which in conjunction with serotyping, pulse field gel electrophoresis (PFGE), determination of antibiotic resistance patterns and plasmid profiling are methodologies that provide significant information for the assessment of *Salmonella* prevalence and epidemiology (Molbak et al., 2006).

2.2 Taxonomy of Salmonella

Salmonella belongs to the family *Enterobacteriaceae* (Guthrie et al., 1991). The genus *Salmonella* contains two species; *S. enterica* and *S. bongori.* Six sub-species are differentiated within *S. enterica* based on their biochemical and genomic characteristics, a roman numeral and a name are used for the designation of these six sub-species as follows: *I, S. enterica ssp. enterica; ii, S. enterica ssp. salamae; iiia, S. enterica ssp. arizonae ; iiib, S. enterica ssp. diarizonae; iV, S. enterica ssp. houtenae, and Vi, S. enterica ssp. indica* (Brenner et al., 2000). With regard to food safety *S. enterica ssp. enterica* is the subspecies of most concern because the strains within these sero-groups are known to cause 99% of *Salmonella* infections in humans (Bell et al., 2008; Brenner et al., 2000). In humans invasive *Salmonella enterica sub-species enterica* (typhus, paratyphus) and non-invasive *Salmonella* are major pathogens causing diarrhea disease.

Table 1: Species and sub-species in the *Salmonella* genus as presented by Card R. (2009)

Salmonella species	**Subspecies (numeral)**	**Number of serovars**
S. enterica	*enterica (i)* *salamae (ii)* *arizonae (iiia)* *diarizonae (iiib)* *houteane (iv)* *indica (vi)*	1,478 498 94 327 71 12
S. bongori		21
Total		2,501

By newer convention, names are retained only for sub-species enterica serovars, and these names are no longer italicized. The first letter is a capital letter “S” followed by the serovar names of sub-species enterica (e.g. *Typhimurium or Montevideo*). At the first citation of the serotype the genus

name is given followed by the word “serotype” or the abbreviation “ser.” Followed by the serotype name. This project follows the abbreviated modern naming system, i.e. *S. Typhimurium* rather than the more complete nomenclature *S. enterica, ssp. enterica serovar Typhimurium*. (Brenner et al., 2000; Molbak et al., 2006). The antigenic formulae are also used to name Salmonella serotypes. This designation includes: (i) subspecies designation (subspecies I trough VI), (ii) O (somatic) antigens followed by a colon, (iii) H (flagellar) antigens (phase 1) followed by a colon, and (iv) H antigens (phase 2, if present) i.e. *Salmonella* serotype IV 45:g,z51:- (Brenner et al., 2000).The nomenclature detailed above is internationally accepted based on recommendations of the WHO Collaborating Center (Molbak et al., 2006).

Table 2: Antigenic formulae of some *Salmonella* serotypes as presented by Card R. (2009)

Serotype	**Serogroup**	**Somatic Antigen (o)**	**Flagella(H)antigen**	
			Phase 1	**Phase 2**
S. *Paratyphi A*	A	1,2,12	A	1,5
S. *Typhimurium* B	B	1,4,5,12	I	1,2
S. *Agona*	B	4,12	F, g, s	-
S. *Derby*	B	1,4,5,12	F ,g	1,2
S. *Typhi*	D	9,12,Vi	C	1,2
S. *Enteriditis*	D	1,9,12	g, m	1,7

2.3 Detection of SalmonellaDetection of Salmonella can be divided into Serological typing, bacterial culture with biochemical tests and molecular tools.

2.3.1 Bacterial culture and biochemical testing

There are four steps for the recovery of injured Salmonella cells from a food matrix. First the pre-enrichment, where buffered peptone water or lactose broth can be used, followed by growth on a non-selective broth. This is followed by enrichment in selective broth, such as Rasspaport-Vasilliadis (RV) broth, Selenite Cysteine Broth (SC), or tetrationate broth (TT). Finally the subsequent isolation is done on either selective Brillant green agar, Bismuth sulfite agar, Hektoen agar (HA) or Xylose Lysine Dextrose agar (Molbak K. et al., 2006). Some strains of Salmonella could have a different reaction to the combinations of inhibitory substances, incubation temperatures, selective enrichment broths and media (Cardinale et al., 2005). Some Salmonella serotypes (*S. Anatum, S. Tennessee, S. Newington* and *S. Senftenberg*) are lactose positive cultures (Bell et al., 2008), for that reason it is important not to rely only on lactose fermantation to distinguish Salmonella from other microorganisms present in the food matrix, but to utilize alternative selective media such as Mannitol Lysine Crystal Violet Brillant Green (MLCB) or Bismuth Sulphite Agar (Kyriakides et al., 2008).

2.3.2 Molecular Identification

The development of DNA-based methods have allowed for novel approaches for detection of Salmonella. The foundation of these methods is the hybridization of two complementary single – stranded molecules (one in the form of a probe, primer, DNA fragment or oligonucleotides developed in the laboratory and the other strand corresponds to the target microorganism) to obtain double- stranded nucleic acid molecules under defined physical and chemical conditions. Other diagnostic tools for Salmonella are the DNA microarrays. These are biochips, which enables hybridization by the presence of immobilized oligonucleotides to a solid base. Results can be analyzed automatically with use of an appropriate device (Molbak et al., 2006). Numerous probes

can be placed on a DNA chip and that number is expanding because of the continued growth of fully sequenced organisms (Molbak et al., 2006).

2.3.3. Kauffman-White scheme for serotyping of Salmonella

Serotyping is an important tool to understand the epidemiology of Salmonella infections, and is frequently used to trace back sources of contamination during an outbreak. The serotyping scheme developed by White and Kauffmann on 1920 was based on the discovery of flagella H antigen, the somatic O antigen and the phase-shift in the H antigen (Molbak et al., 2006). The Kauffmann-White (KW) method, used worldwide, is considered the gold standard for identification of Salmonella serotypes..

The scheme used worldwide for serological identification of Salmonella serovars was first proposed by White and expanded by Kauffman (Popoff et al., 1987). The list of 2,501 *Salmonella* serotypes is maintained and annually updated by the World Health organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (Brenner et al., 2000). The KW scheme is based on the antigenic structure of *Salmonella* serotypes (Wong et al., 2002). The antigenic properties and variations of the O (surface polysaccharide) and H (*flagellar*) antigens from each serovar are summarized and described in what is known as the antigenic formulae (Mortimer et al., 2004; Wattiau et al., 2008). The structure of each microbial cell is dependent of a variety of antigenic molecules, which are at the time dependent of many determinant groups (chemical groups). Thus it is the chemical make-up and the arrangement of these determinant groups what assign the immunological specificity of the antigen (Molbak et al., 2006).

The cross absorption of antisera is used to reveal the antigenic structure of *Salmonella* (Helmuth et al., 2000; Jones et al., 2000). The composition and structure of polysaccharides, which constitute a part of the structure of the cell surface, allow for recognition and differentiation of O antigens (Molbak et al., 2006). In the KW scheme O antigens are indicated in brackets when they are easily modified by mutation, otherwise they are underlined when these factors are determined by bacteriophages or plasmids (Helmuth et al., 2000; Jones et al., 2000). H antigens are present in the flagella, they are composed of protein subunits called *flagellin*, that are typically diphasic and thought to help the bacteria to survive host immune responses (Helmuth et al., 2000; Jones et al., 2000). A capsular polysaccharide is found in some serovars (*Typhi, Paratyphi* C and Dublin) is termed "The virulence (Vi) antigen". This factor first needs to be heated at 100 °C for 60 min to remove the capsule; otherwise it would not be agglutinable with anti-O antiserum (Helmuth et al., 2000; Jones et al., 2000).

Serological typing of *Salmonella Enterica* serovars requires, over 150 O and H antigens and more than 250 antisera (Cai et al., 2005; Wattiau et al., 2008). The problem with this conventional method is that it is laborious, time consuming, and cannot differentiate within serovars. It also depends on the availability of hundreds of antisera, needs highly trained personnel, consumes high volumes of reagents, and a minimum of three days is required to identify a serotype (Alvarez et al., 2004; Cai et al., 2005). Food safety objectives from regulatory agencies encourage food industry manufacturers to establish full identification of Salmonella serotype to assist with traceability in the food processing (Molbak et al., 2006). In addition, the capability to serotype or fingerprint is of importance for surveillance, inspection, and investigation of outbreaks. Salmonella subtyping can be accomplished by biotyping, phage typing, antibiotic resistant patterns, PFGE, and ribotyping (Berge et al., 2004).

2.3.4 *Salmonella* Incompatibility group typing

A formal scheme of plasmid classification is based on incompatibility (Inc) groups (Novick et al., 1987). The procedure for incompatibility grouping is based on the introduction, by conjugation or transformation, of a plasmid of an unknown Inc group into a strain carrying a plasmid of a known Inc group. If the resident plasmid is eliminated in the progeny, the incoming plasmid is assigned to its same Inc group (Datta et al., & Hedges et al., 1971). Plasmids with the same replication control are incompatible Q, whereas plasmids with different replication controls are compatible. On this basis two plasmids belonging to the same Inc group cannot be propagated in the same cell line (Couturier et al., 1988; Datta N. & Hedges et al., 1971). Inc group identification has been frequently used to classify plasmids. The method has been an important tool to trace the diffusion of plasmids conferring antimicrobial resistance and also to follow the evolution and spread of emerging plasmids (Anderson et al., 1977).

In 1988 Couturier et al. developed a new method for the identification of the major replicons of plasmids circulating among the *Enterobacteriaceae*. This method was based on hybridization with 19 DNA probes that recognize different basic replicons (Couturier et al., 1988). The necessity of tracing plasmids conferring drug resistance prompted us to develop an inc/rep PCR-based typing method. In this method, 18 pairs of primers were designed to perform 5 multiplex- and 3 simplex-PCRs, recognizing the FIA, FIB, FIC, HI1,HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons, representative of the major plasmid incompatibility groups circulating among the Enterobacteriaceae (Couturier et al., 1988).

2.4 Salmonella Infections

Infections caused by *Salmonella* serotypes can produce enteric fever, gastroenteritis, and bacteremia or septicemia conditions (Guthrie et al., 1991; Matthews et al., 2008). Many animals, especially pigs and poultry, are colonized showing no clinical illness hence maintaining carrier status. In this condition, carrier animals continue shedding Salmonella and thereby acting as sources of human contamination and infections (Kalule et al., 2012; Molla et al., 2003).

None-Typhoid Salmonellosis organisms are etiological agents that cause diarrheal and systemic infections in humans, arising mostly from consumption of contaminated food (Yang et al., 2010). Animal source food, fruits and vegetables have been implicated to be the main sources of *Salmonella* infections in man (Guentzel et al., 2008).The disease is often self-limiting in healthy individuals. Symptoms appear 8 to 72 hours after ingestion, with non-bloody diarrhea and abdominal pain which disappear within five days. Infections caused by NTS serotypes can also evolve into systematic infections followed by chronic conditions (Monteville et al., 2008. & Matthews et al., 2008).

2.5 Epidemiology of Non-typhoid Salmonella infections

Salmonella is one of the leading cause of foodborne illness worldwide (Cardinale et al., 2005) causing Salmonellosis and several other infections in humans and animals.

Globally, non-typhoidal Salmonellosis accounts for approximately 80.3 million cases of human infections annually (Tinega et al., 2016). Estimates of Salmonellosis due to consumption of pork or pork products is difficult to determine but, it ranges from < 1% to 25% (Tinega et al., 2016). These invasive pathogens colonize intestinal mucosal surface but, they are self-limiting in health

individuals due to a noble immunity. Non-typhoidal Salmonellosis *is* of concern in humans in sub-Saharan Africa, and this is partly due to the high number of immune-compromised persons. All non-typhi *Salmonella spp*. (over 2500 serovars) are considered as human pathogens (WHO, 2013). The NTS is more common among children, the elderly and the immune-compromised persons (Shaw et al., 2008). In Uganda, with the advent of the Human Immune Deficiency Virus (HIV) infections, many people are now highly susceptible to clinical and life-threatening NTS with increased prevalence in persons with very low CD4+cell counts (Nasinyama et al., 2016).

In pigs, clinical *Salmonellosis* is considered uncommon (Kranker et al., 2003) and only a few serovars namely *Salmonella enterica sub-species enterica* serovar *Cholera-suis (S. Cholerasuis), S. Typhimurium, S. Enteritidis and S. Derby* have been implicated in clinical disease (Fedorka-Cray et al., 2000). In piglets and growing pigs, Salmonella infections may cause enterocolitis, septicemia and death. However, sub-clinical infections are common (Aragaw et al., 2007; Vigo et al., 2009; Wong et al., 2002) and therefore, pork and pork products are considered to be among the major sources of NTS for humans world over (EFSA., 2008). With the increased consumption of pork and pork products in Uganda there is need for more surveillance and inspection programs to minimize cases of food borne illnesses.

In industrialized countries, *Salmonella* surveillance and monitoring systems are implemented to identify specific serotypes involved and to facilitate better management of disease outbreaks (Olsen et al., 2001). There is limited data in African developing countries largely due to inadequate funding for research, lack of proper testing laboratories, and absence of qualified personnel to isolate and characterize *Salmonella* (Scallan et al., 2011). Lack of such surveillance and inspection programs in the country, there is little data available to support any policy formulations in the

country and to give a good estimate of the burden as it is in developed countries. Although developed countries of United States of America (USA) and Europe have seen tremendous gains in the management and control of *Salmonellosis*, it still remains a major problem in developing countries in Africa, Asia and South America, and acts as a source of infection to foreigners returning from such countries (Santos et al., 2001).

Preventing *Salmonella* from contaminating food during the farm to table process remains challenging in African countries, and even up today there is limited efforts towards this cause. In the industrialized countries like the USA however, several interventions have been designed by governments. The food industry especially pork poultry and processors, are highly regulated by government monitoring programs (Mead et al., 2000; Mead et al., 1999).

Pre-requisite programs and Hazard analysis and critical control points (HACCP) plans are successful strategies already adopted by the meat industry. Interventions, which demonstrate effective reductions in the occurrence and levels of pathogenic bacteria at different processing steps, have been included in production lines to keep critical points under control. For example, combinations of temperature and pressure on sprays with or without bactericides at different levels, steam with or without vacuum, irradiation, pulsed electric fields, high pressure, ultraviolet light and microwaves are some of the decontamination treatments being used for decontamination of pork and poultry carcasses (Bautista et al., 1997). Some of the chemical treatments include: dioxide, acidified sodium chlorite, ozone, organic acids, trisodium phosphate (TSP) and cetylpyridinium chloride (CPC) (Kyriakides et al., 2008; Meneses et al., 2010; Petry et al., 2013). Surveillance and inspection programs have played a major role in the reduction of cases of food borne illnesses something which developing countries like Uganda should adopt.

2.6 **Antibiotic resistance in NTS strains**

Resistance to antimicrobials and particularly multidrug resistance is an emerging problem in Enterobacteriaceae for developing and developed countries (White et al., 2005). Antibiotic resistance has an important social and economic impact, and there is a need for stronger scientific and public health efforts to better regulate, control and monitor the use and abuse of antimicrobials (Helmuth et al., 2000; Jones et al., 2000). This is a serious public health concern in human and veterinary medicine (Su L et al., 2004). Resistant microorganisms have emerged as a result of improper use of antibiotics in human health as well as in agricultural practices (Khachatourians et al., 1998). For example, in USA it has been reported that most of the antibiotics produced are fed to farm animals as growth promoters and to obtain a better meat to feed ratio (Goldman et al., 2004). In the pork and poultry industry low levels of bacitracin, chlortetracycline, erythromycin, lincomycin, neomycin, oxytetracycline, penicillin, streptomycin, tylosin or virginiamycin are administrated in each ton of feed (Khachatourians et al., 1998). Over the time these low doses of antimicrobials confer the ability of microorganisms to evolve mechanisms of defense, therefore making themselves less susceptible to the effect of the drug and contributing to treatment failure.

Salmonella has been widely documented to possess resistance to several antibiotics used in medical treatment. In fact antibiotic-resistant *Salmonella* accounted for an annual mortality estimate of 4,760 deaths in the USA alone (Khachatourians et al., 1998)..The multidrug resistant salmonellosis is an increasing problem (Leekitcharoenphon et al., 2013) generally attributed to the unsupervised usage of antimicrobial agents for growth promotion and nutritional enhancement in livestock animals such as pigs (Rayamajhi et al., 2008). Antimicrobials such as sulphonamides, trimethoprim, fluoroquinolones, aminoglycosides, chloramphenicol and tetracycline are broadly

used for both livestock and human treatment (Su L et al., 2004; Tinega et al., 2016). Invasive NTS in humans is treated by the use of antimicrobials. In Uganda, the most commonly used drugs are chloramphenicol, ciprofloxacin and nalidixic acid (Kalule et al., 2012). Lately, cases of drug-resistant NTS spp. have been reported in a number of countries in Africa (Kariuki et al., 2006). With increasing and rampant use and misuse of antibiotics in developing countries (Byarugaba et al., 2004; Dowell et al., 2004), this situation is bound to worsen.

Salmonella Typhimurium and *Salmonella Heidelberg* are ranked first and second respectively in multidrug resistance and are among the most commonly-isolated serovars from non-clinical, non-human sources (Patchanee et al., 2008). Four different antimicrobial resistance patterns were found in a study where *Salmonella Heidelberg* isolates from swine were tested for resistance to a panel of 12 antibiotics (Patchanee et al., 2008). All isolates showed resistance to amoxicillin-clavulic acid, amikacin, ceftrixone, ciprofloxacin, cephalothin, and gentamicin (Patchanee et al., 2008). *S. Typhimurium* and *S. Muenchen* isolates from swine have shown resistance to ampicillin, chloramphenicol, amoxicillin, clavulic acid, kanamycin, streptomycin, sulfisoxazole, and tetracycline in Vietnam (Patchanee et al., 2008).

CHAPTER THREE:

3.0 MATERIALS AND METHODS

3.1 Study design and study site

This was a retrospective cohort utilizing samples archived from an earlier study done by Heilmann et al., 2015 and kungu et.,2014 al. this study was part of the safe food fair food project of the international livestock research institute which was a five year project. The work started with Kungu et al who mapped pork outlets in Kampala in an attempt to understand food dynamics in informal market systems. The pork outlets mapped by kungu et al were used in afollow up study under the same project by Martin Heilmann (Heilmann et al., 2015) who was studying food hygiene in pork outlets using Salmonella as an indicator pathogen and the role of flies as vectors of disease. Martin Heilmann randomly selected 77 pork joints from those mapped by Kungu in 2014. From the 77 pork outlets, martin looked the potential risk factors for salmonella contamination of food and these included; flies, vegetables salads, raw pork, cooked pork, workers, utensils, water. All together identifying 9 items to be sampled for Salmonella testing from each pork outlet. 673 samples were collected for laboratory analysis at the Central diagnostic laboratory using the ISO 6579:2002. All together 55 isolates were obtained from Martin Heilmanns study that have been used in this study.

3.2 Study population and sample size

The study population composed of 55 *Salmonella* isolates (isolated from raw pork, roasted pork, cabbages, onions, tomatoes, water, and fly mid-guts) obtained from the previous study entitled **"Flies as vectors for Salmonella spp. and their control in pork butcheries in Kampala, Uganda – A contribution to improved public health"**. the *Salmonella* isolates were stored at the Central Diagnostic Laboratory College of Veterinary Medicine Animal Resources and Biosecurity (COVAB), Makerere University.

3.3 *Salmonella* Isolate retrieval

Salmonella isolates preserved at -20 at CDL-COVAB on Muller Hinton slants in cryotubes were transported to Frei University of Berlin (FUB) by DHL courier services following standard biological transportation recommendation (dry ice transportation).

In the laboratory at Berlin, the isolates on Muller Hinton were stored temporary at 4°C. These were later cultured in LB broth and incubated overnight at 37°C.

3.4 Confirmation of Salmonella isolates

DNA was extracted from the isolates by Chelex method and amplified using salmonella species specific primers to confirm the species before serotyping and antimicrobial resistance testing.

3.5 Antimicrobial susceptibility testing of Salmonella isolates

Antimicrobial susceptibility testing was performed using disc diffusion method; four plates of Muller Hinton agar were prepared for each of the 55 isolates. 6 pure colonies of each isolate were dissolved in 5mls of sterile PBS solution to form a bacteria solution. 100µl of this bacterial solution was applied to each of the 4 plates and spread on the media uniformly using a glass spreader. A

maximum of six antibiotic discs were applied on each of the four plates a distance of between 24mm to 1cm. The following antibiotics were used in the study; ampicillin (AP; 10µg), amoxicillin-clavulanic acid (AMC; 20/10µg), ampicillin-sulbactam (SAM; 10/10µg), piperacillin (PRL; 100µg), piperacillin-tazobactam (PTZ; 100/10µg), cefazolin (CZ; 30µg), cefepime (CPM; 30µg), cefotixime (CTX; 30µg), cefoxitin (FOX; 30µg), ceftazidime (CAZ; 30µg), cefuroxime sodium (CXM; 30µg), cephalothin (KF; 30µg), imipenem (IMI; 10µg), meropeme (MEM; 10µg), amikacin (AK; 30µg), gentamicin (GM; 10µg), tetracycline (T; 30µg), ciprofloxacin (CIP; 5µg), levofloxacin (LEV; 5µg), ofloxacin (OFX; 5µg), chloramphenicol (C; 30µg), and sulfmethoxazole-trimethoprim (SXT; 23/1.25µg) (Mast, Bootle, GB). The plates were incubated at 37°C overnight, after which the results were recorded for each antibiotics on a particular isolate by measuring the zones of clearance around the disc. The results were recorded as Susceptible, Intermediate, and Resistance depending on the diameter of the zone of clearance. Corresponding break points were obtained using guidelines set by the Clinical and Laboratory Standards Institute (CLSI). The control strains used were Enterococcus Fecalis 700802 and E.Coli control strain ATCC 25922 from the reference laboratory for foreign biological risk in Berlin (BFR)

3.6 Salmonella serotyping using O and H antigens

All isolates were sub cultured on STI, from each of the subculture one colony was cultured on selective agar (Rambach and BPLS). The Biochemical assays for identification of *Salmonella* we used: carbohydrates (O/F) test, glucose utilisation, lactose hydrolysis, Hydrogen sulphide gas production gas production, lysine carboxylase test, urease hydrolysis, indole test, VPR test, motility test. The isolates were then typed using the O-antigen and the H-antigen panel were colonies of salmonella are emulsified in antisera O or H (produced in house at BFR reference lab)

contained in U-shaped flat bottom 96 well plates. Positive reaction are observed by formation of an agglutination.

3.7 Incompatibility group typing of Salmonella isolates

Genomic DNA was extracted from the salmonella isolates using the chelex method as described by Carroteli et al., 2005; briefly three to five Salmonella colonies were washed in 500 µl of Tris-EDTA buffer and centrifuged at 10,000 × g. The pellet was resuspended in 500 µl of 5% Chelex in water and incubated under constant shaking (Eppendorf) for 1 h at 56°C, 700 rpm, later incubated at 95°C, 700 rpm for 20 minutes after which it was centrifuged 10,000 × g. Then the supernatant containing the genomic DNA was collected and used for PCR. The PCRs were performed according to Carattoli et al 2005 in which 18 pairs of primers (See appendix I) designed to perform five multiplex and three simplex PCR reactions were used, recognizing the following 18 incompatibility groups: FIA, FIB, FIC, HI1, HI2, I1-I$^{\gamma}$, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA.

3.7 Data management and analysis

Data was entered into Microsoft Excel, version 10, for descriptive analysis. The antimicrobial resistance data was entered as Resistant (R), intermediate (I) or susceptible (S). Data from molecular analysis was captured using a gel documentation system and later exported to excel for analysis with all the other data.

3.8 Ethical considerations

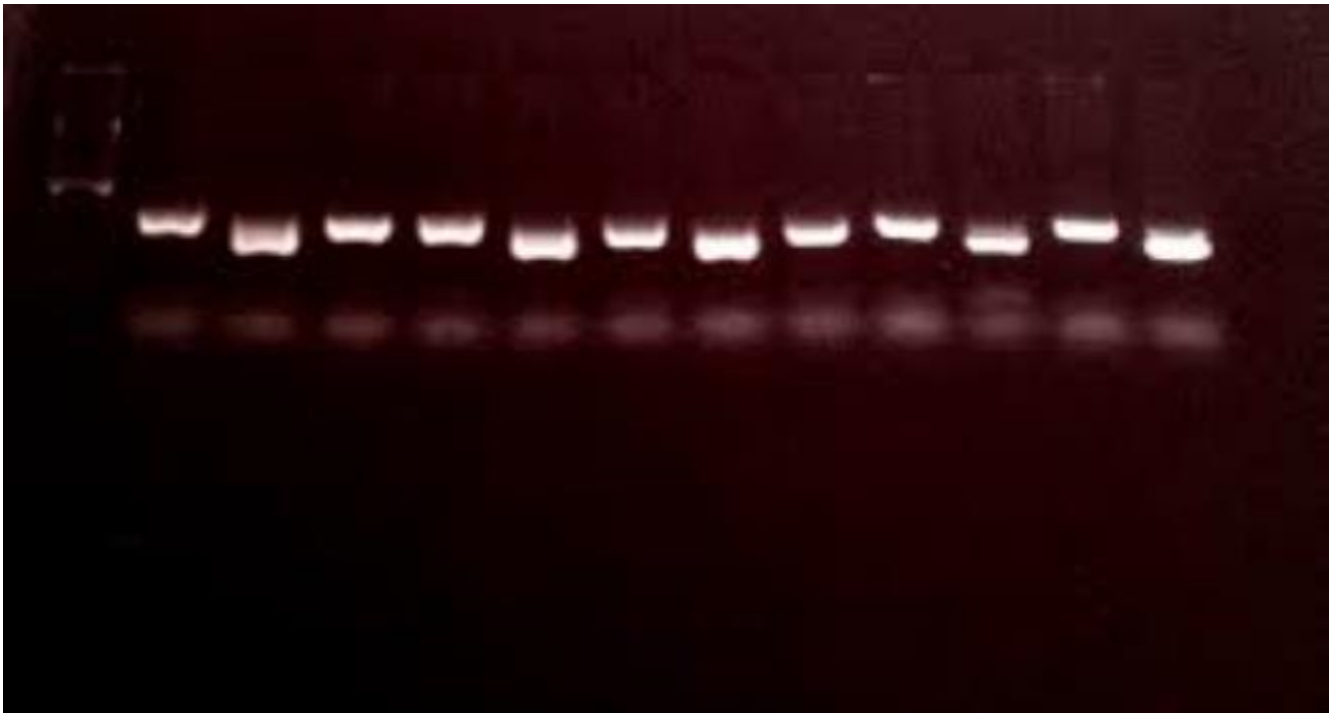
Permission to proceed with the study was obtained from the Department of Public health at COVAB and CDL/ILRI who own the salmonella isolates. Institutional approval for the study was obtained from the Makerere University College of Veterinary Medicine Institutional Review Board (IRB) and the Uganda National Council for Science and Technology.

CHAPTER FOUR: RESULTS

4.1 Serotypes of Salmonella isolates obtained from pork outlets in peri-urban Kampala

A total of 55 isolates were serotyped and identified as *S Enterica. Enteritidis* (33/55; 60.0%), *S.Enterica Offa* (6/55; 10.8%), *S. Arechavaleta* (4/55; 7.3%), *S. Gallinarum* (4/55; 7.3%), *S. Zanzibar* (4/55; 7.3%), *S. Kampala* (3/55; 5.4%), and *S. Saintpaul* (1/55; 1.8%).(See table 1)

PCR results for Salmonella confirmation



Bands of amplified Salmonella DNA under UV light in gel documentation

Table 3: *Salmonella enterica subsp. enterica* serotypes by type of sample identified in different substrates from 77 pork outlets in Kampala, Uganda (where n= number of isolates obtain from each of the substrates in the table is a fraction of total number of isolates in the study)

Serotype	Raw pork n(%)	Roasted pork n(%)	Fly midgut n(%)	Cabbage n(%)	Tomatoes n(%)	Onions n(%)	Water n(%)
S. Arechavaleta	3(75.0)	0	0	0	0	1(25.0)	0
S. Kampala	0	0	0	0	0	0	3(100.0)
S. Offa	0	0	0	2(33.3)	2(33.3)	0	2(33.3)
S. Enteritidis	19(57.6)	1(3.0)	12(36.4)	0	1(3.0)	0	0
S. Gallinarum	0	0	0	2(50.0)	2(50.0)	0	0
S. Saintpaul	0	0	1(100.0)	0	0	0	0
S. Zanzibar	0	0	2(50.0)	0	0	0	2(50.0)
Sub-total	22(40.0)	1(1.8)	15(27.3)	4(7.3)	5(9.1)	1(1.8)	7(12.7)

S. Arechavaleta was isolated from raw pork and onions only, *S. Kampala* was isolated from water samples only. S. *Offa* was isolated from vegetables and water, *S. Enteritidis* was found in flies, raw pork , roasted pork, tomatoes, *S. Gallinarum* was found in vegetables, *S. Saintpaul* was in flies while *S. Zanzibar* was found in flies and water samples

4.2 Antimicrobial resistance patterns of *Salmonella enterica subsp. Enterica* isolates

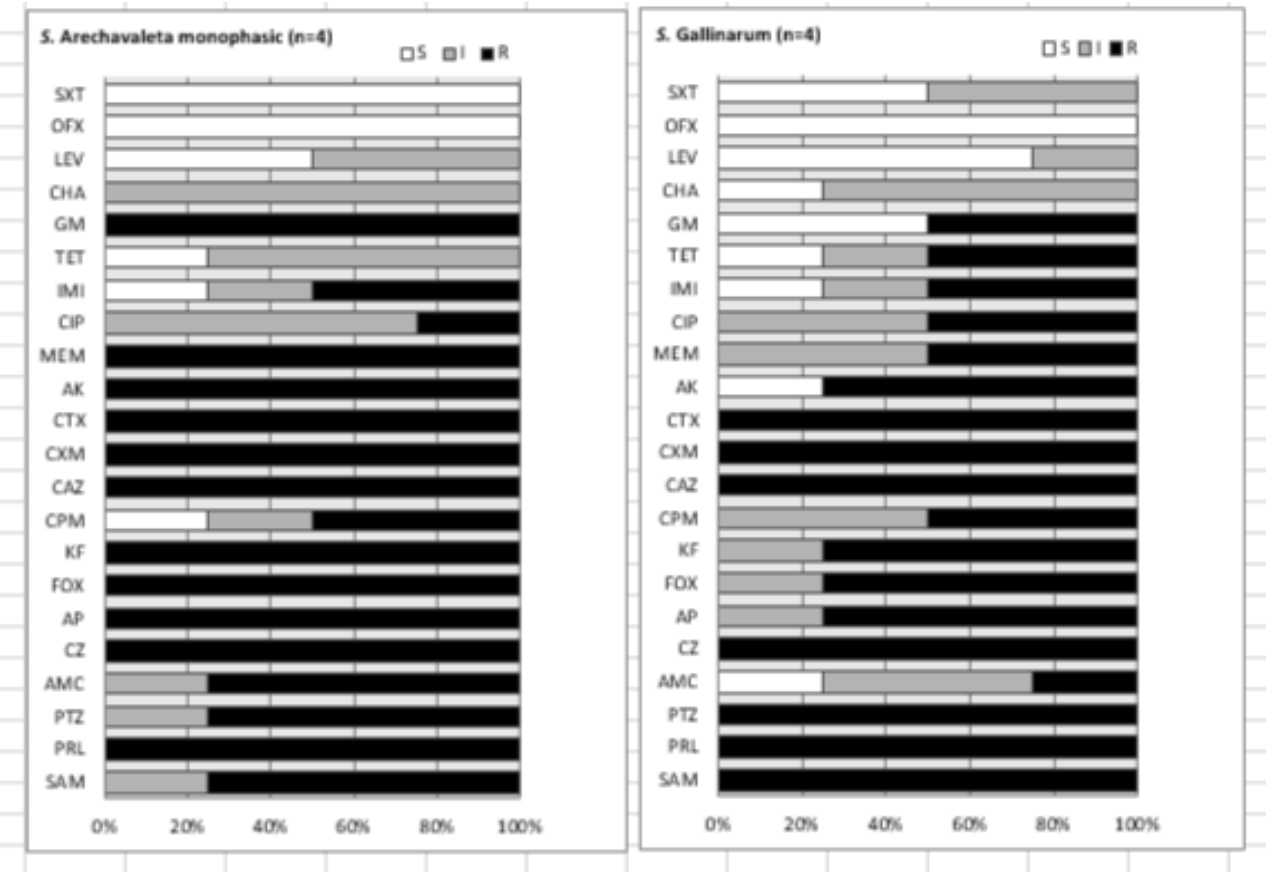
The highest antimicrobial resistance was found for cefazolin (94.5%), cefotixime (92.7%), gentamicin (89.1%), ceftazidime (87.3%), amikacin (85.5%), and piperacillin (85.5%). Resistance to tetracycline was low at 16.4%, similar to chloramphenicol (16.4%). Levofloxacin, ofloxacin, and sulfmethoxazole-trimethoprim showed very low resistance of 1.8%. Most resistant strains were found in isolates from raw pork, followed by those from flies. Isolates from tomatoes on the other hand, had the lowest number of resistant strains (Figure 1). See Table 2 below.

Figure 2: Distribution of antimicrobial resistance among 55 isolates of *Salmonella enterica subsp. enterica* serovar's *Enteritidis* and *Offa* obtained at pork outlets in Kampala, Uganda

For *S.Enteritidis* highest resistance was shown to Cephazolin, Cefotaxime, Gentamycin and Amikacin as indicated above while least resistance was shown towards Sulfamethoxazole, Oflaxacin, Tetracycline and ciprofloxacin.

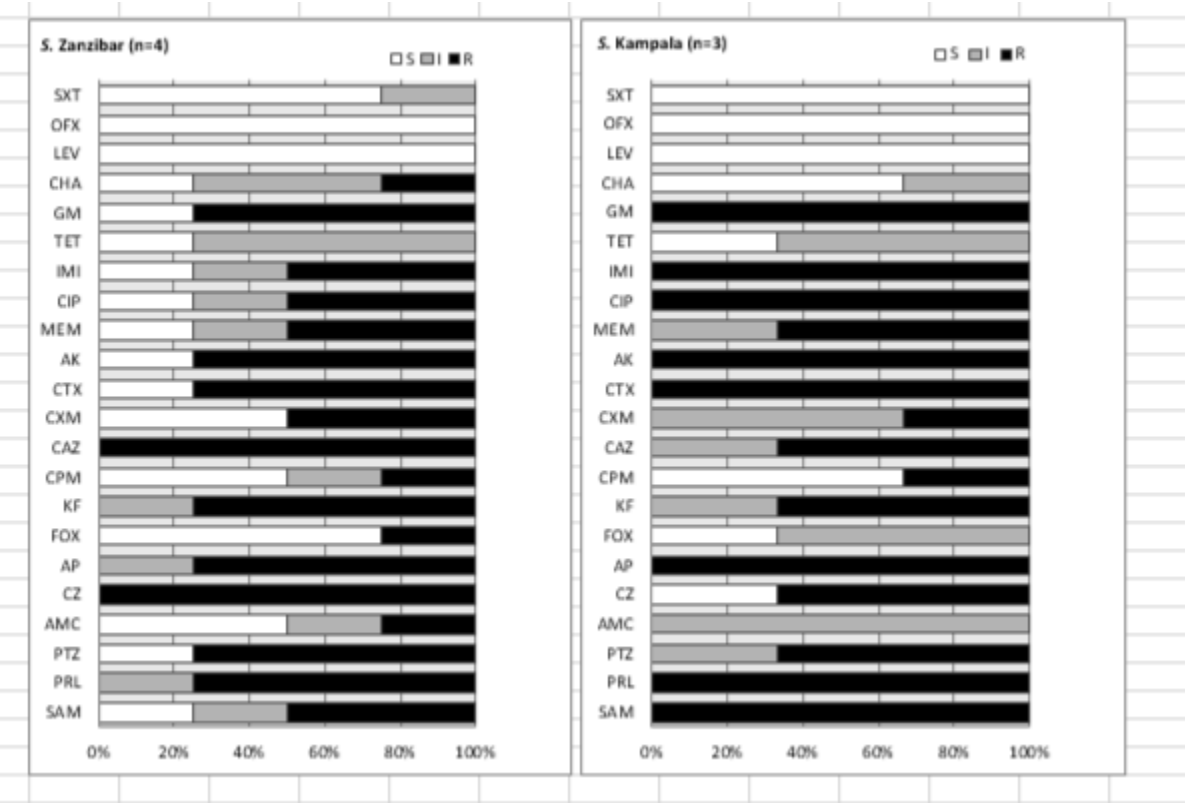
For S. Offa, highest resistance was shown towards Gentamycin and Ampicillin Sulbactam antibiotics. Generally there was greater resistance shown by the *S.Enteritidis* strains compared to Offa strains.

Figure 3: Distribution of antimicrobial resistance among 55 isolates of *Salmonella enterica subsp. enterica* serovar's *S.Arechavaleta* and *S.Gallinarum* obtained at pork outlets in Kampala, Uganda



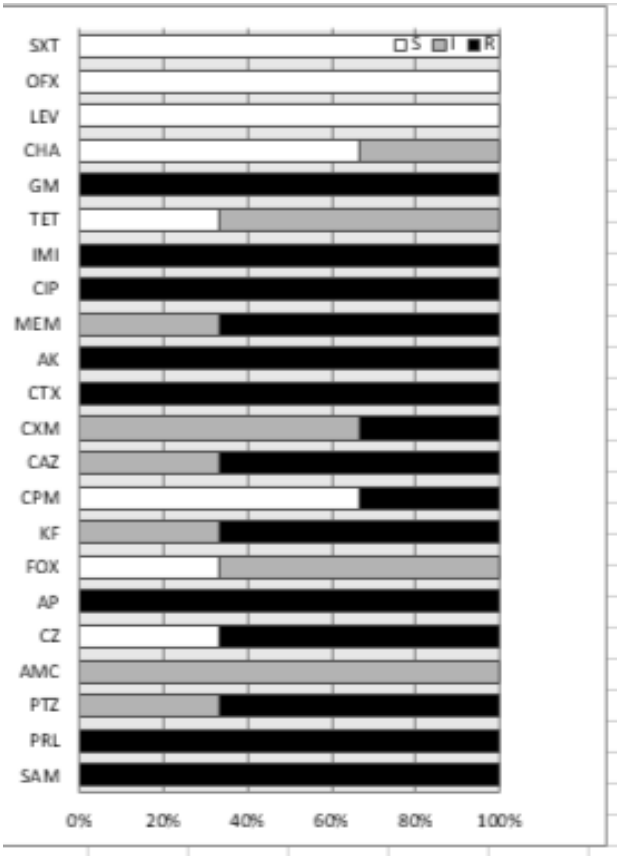
S.Arechavaleta was completely resistant to 11 antibiotics as shown above in the figure but was completely susceptible to Sulphamethoxazole, Oflaxacin. *S.Gallinarum* was completely resistant to 7 antibiotic discs as shown in the figure but was susceptible to Ofloxacin antibiotic. Generally S.Arechavaleta and *S.Gallinarum* were more resistant compared to *Offa* and *Enteritidis*

Figure 4: Distribution of antimicrobial resistance among 55 isolates of *Salmonella enterica subsp. enterica* serovar's *S. Zanzibar* and *S. Kampala* obtained at pork outlets in Kampala, Uganda



S. Zanzibar showed high resistance to Cefazolin and ceftazidime but susceptible to Oflaxacin and Levofloxacin while *S. Kampala* showed highest resistance towards Gentamycin, Ampicillin-Sulbactam, Piperacillin, Cefotixime Amikacin and Imipinem. Generally Isolates of *S. Kampala* showed a lot of resistance compared to *S. Zanzibar*.

Figure 4: Distribution of antimicrobial resistance among 55 isolates of *Salmonella* enterica subsp. enterica serovar's *S. SaintPaul* obtained at pork outlets in Kampala, Uganda



S. SaintPaul showed complete resistance towards Gentamycin, Imipinem, ciprofloxacin, Cefotixime, Piperacillin, Ampicillin, Ampicilin-Salbuctam antibiotics. However these strains were susceptible to Oflaxacin, Levofloxacin, Chloramphenicol and Cefepime.

Among all the serovars high resistance was shown towards Gentamycin, Ceftazidime, Amikacin and Piperacillin drugs while least resistance was shown towards Ofloxacin, Levofloxacin and Sulfamethoxazole-trimethoprim antibiotics.

4.3 Plasmid profiles in the *Salmonella* strains

The inc/rep typing detected the presence of FIA, W, FIC, P, FIB, Y replicons in 54 of the 55 (98.2%) isolates. Two isolates, both *S. Enteritidis* isolated from flies, contained none of the replicons tested. FIA, FIB replicons were identified in isolates of raw pork source, replicon types Y and P were exclusively identified in isolates from flies' mid guts and water. Replicon types FIC and W were identified in isolates from all sources (raw pork, roasted pork, fresh vegetables, water and flies). Several replicon types were identified in the isolates; only two of the isolates did not present any plasmids, the FIC replicon of amplicon size 262 bp was present in 35 of the 55 isolates (63.6%), Forty percent 40.0% (22/55) of the isolates contained the replicon type FIA of size 462 bp. Most isolates that showed presence of plasmids had more than one plasmid present, with many isolates presenting five different plasmids. See Table 3 below.

Table 3: Plasmids identified by PCR-based replicon typing in 55 *Salmonella enterica* isolates from 77 pork outlets in Kampala, Uganda, presented by substrate

		Substrate			
Plasmid (inc/rep group)	**Total** n = 55 (%)	**Pork** n = 23 (%)	**Flies** n = 15 (%)	**Raw vegetables** n = 10 (%)	**Water** n = 7 (%)
FIA	20 (36.4)	18 (78.3)	0	2 (20.0)	0
FIB	10 (18.2)	9 (39.1)	0	1 (10.0)	0
FIC	41 (74.5)	19 (82.6)	9 (60.0)	6 (60.0)	7 (100.0)
P	19 (34.5)	4 (17.4)	10 (66.7)	0	5 (71.4)
W	42 (76.4)	20 (87.0)	9 (60.0)	8 (80.0)	5 (71.4)
Y	15 (27.3)	0	10 (66.7)	0	5 (71.4)

There was an observed significance difference in the distribution of plasmids among the different the isolates with the W, FIC being more prevalent.

CHAPTER FIVE: DISCUSSION

This study was a retrospective cohort utilizing fifty five 55 salmonella isolates from a previous study, the aim of the study was to determine the serotypes, the antimicrobial resistance profile and to determine the presence of any resistant plasmid. Seven different serotypes were identified, multi drug resistance (resistance to more than one antibiotics used) was identified at 98%, and presence of shared plasmids was observed.

Seven serovars were identified; *S. Arechavaleta, S. Kampala, S. Offa, S. Enteritidis, S. Gallinarum, S. Saintpaul, S. Zanzibar*. The most predominant serotype being *S. Enteritidis* , this is of public health importance as S enteritidis is one the mojor causes of non-Typhoid Salmonella cases in Humans. Globally, non-typhoidal salmonellosis accounts for approximately 80.3 million cases of human infections annually most of these occurring children, elderly and immune compromised populations. With the increased population of people living with HIV in Uganda, containing the circulation of this serovar becomes of importance. We identified *S Kampala* and *S Zanzibar* which were reported by Ikwap et al 2014 using another cohort, this shows that these two serovars are commonly circulating within the Ugandan environment. Another important finding is the presence of *S. Arechavaleta var. monophasic* which has been deleted from the WHO reference list in 2007 but had been listed in prior editions, this indicates more serovars not known or deleted by WHO but yet circulating in some developing countries were research is limited and there are no surveillance programs. More research and surveillance programs on salmonella need to be developed in developing countries.

Data from the sensitivity studies showed multidrug resistance in 54/55 (98%) of our isolates. The observed high prevalence of multidrug resistance shows an increase in antimicrobial burden in the study area which could have risen from personnel contamination, environmental contamination or

irrational antibiotic use at the farms (Goldman et al., 2004; Hombach et al., 2014; Kalule et al., 2012; Khachatourians et al., 1998; Roesel et al., 2014). However with regard to piggery, several studies suggest low antimicrobial use in the backyard farms where most Ugandan pork is produced (Dione et al., 2014; Ikwap et al., 2014) and resistance in pigs may most likely arise from environment contamination and feed processing companies with new feed promoters like big pigs which are being used in intensively managed pig farms. in some instance pigs a fed on waste products and feeds from intensively managed poultry farms and Aquiculture farms yet there is increased drug use in these enterprises which can increase the drug selective pressure that can spill over into pig enterprises and the possibility of introduction of resistant bacterial strains in pig enterprises. Additionally, it is clear that drugs for human use (ceftazidime, cefuroxime, cefotixime, meropeme, amikacin) are showing resistance yet they are not lincensed for animal use, this can be as a result of environmental or slaughter personnel contamination of pork during the preparation process.

The Inc. group typing Presence showed presence of two shared plasmids FIC and W which were shared among the different serotypes. This gives an indication of bacterial mounting up response to a common pressure in the environment. Studies by (Kalule et al., 2012) reported the presence of five plasmids and different bacteria showing presence of more than one plasmid, a finding similar to our observation. Further studies are required to link the high resistance to presence of resistance genes carried by these shared plasmids but for now we cannot conclude that these plasmids are responsible for the observed multidrug resistance but we cannot rule this out either..

CHAPTER SIX: RECOMMENDATIONS

1. There is a potential threat of non-typhoid salmonellosis in Kampala due to the high prevalence of *S Eneteritidis*, there is also a high possibility of many interesting serovars that are important to WHO that are possibly circulating in Kampala hence the need for surveillance programs
2. Antimicrobial resistant is an emerging public health threat and very urgent intervention have to be engineered to minimize the antibiotic pressure in the environment. The high antibiotic pressure in the environment has led to pathogen adaptation and they are doing so by acquiring plasmid which a most likely to carry resistance genes.
3. More studies are necessary to understand the importance of shared plasmids in these bacteria and the role of these shared plasmids in promoting antibiotic resistance.

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Appendix

Appendix 3.0: Laboratory procedures

Recovery of cultures/isolates:

The isolates have been preserved in 10% skimmed milk. These will be recovered using Brain Heart Infusion broth, 200ul of the skimmed milk will be pipetted in 6mls of brain heart infusion broth in glass test tube. This will be incubated at 37°C for 24hrs. These will be checked under a microscope to investigate viability of the isolates and detect any possible contamination. The isolates will then be cultured on DHL agar, and a colony will be stabbed on Muller Hinton and incubated for growth. The growth on Muller Hinton stab cultures will be sealed and stored for transportation to Berlin.

Sample transportation to Berlin:

A material transfer agreement will be obtained from the national council of science and technology. The isolates will be packed in biological packaging material on dry ice for transportation to Frei University Berlin via DHL courier services.

Molecular identification of the isolates:

This will be carried out using salmonella specific primers. DNA will be extracted from the isolates using DNA Chelex method of extraction. The resultant DNA will be stored at -20°C for molecular work using specific salmonella primers.

Drug sensitivity Tests:

Drug sensitivity tests will be done using LB agar and 25 antibiotics. The isolates will be cultured on LB agar and two colonies will dissolved in sterile saline solution. From this solution 100ul will be transferred onto LB media and the LB plate will be spread with this culture solution. Five plates of LB media will be prepared for each isolate so as to accommodate 22 antibiotics.

After adding the antibiotic discs, the plates will be incubated at 37°C for 24hrs. The results will be read by measuring the zones of inhibition at the end of the 24hrs.

The plasmid profile analysis will be done according to Caroteli and 18 pairs of primers will be used to identify 18 different plasmids present in Salmonella. The results will be initially stored in gel documents within the laboratory then transferred to excel.